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<b>(54) Title:</b> DNA MARKERS FOR PIG LITTER SIZE  <b>(57) Abstract</b>  Methods for screening pigs to determine which are more likely to produce larger litters and/or are less likely to produce larger litters are provided, based on identification of OPN alleles present in a sample of pig genomic DNA. Kits for use in such methods are also provided.		

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## DNA MARKERS FOR PIG LITTER SIZE

The present invention relates to methods of screening pigs to determine the presence or absence of osteopontin (OPN) alleles associated with increased litter size, to the use of such methods in predicting litter size in pigs and to kits for carrying out such methods.

Meat production and animal breeding efficiencies could be improved if it were possible to increase animal litter sizes. The same output of livestock could be derived from fewer parent animals, thus providing decreased production costs. In addition, animal breeding organisations would benefit from the potential to screen more offspring for those with improved genetics. However, litter size is very difficult to select for conventionally as it is limited to one sex and is heavily influenced by non-genetic factors (heritability, a measure of the fraction of the phenotypic variation that is due to genetic differences is approximately 0.1 for litter size in the pig).

One approach to improving litter size might be to introduce beneficial genes into production lines from breeds which have significantly higher litter sizes. However, quantitative genetics suggests that complex traits such as litter size are controlled by a large number of genes each having a small effect on the trait. If this is true, genetic progress through selection of complex traits is likely to be very slow. An alternative view is that, although many genes are involved in complex traits, a few of the genes involved (major genes) have large effects on the trait. If this alternative view is true, then genetic progress of such traits could be

rapid, provided that it is possible to identify and select for beneficial alleles of relevant major genes. Since the advent of genome mapping, it has become possible to identify genes affecting quantitative traits (quantitative trait loci, QTL) by looking for associations between the trait and molecular markers distributed evenly across the genome of animals for which maps are available. Importantly, for selection purposes, the heritability of such marker phenotypes is 1.0.

The Chinese Meishan breed of pig is known to produce about 4 extra piglets per litter than the most prolific European breeds. Genes for prolificacy (litter size) from this breed would be of great value in programmes aimed at increasing the litter size of commercial Western pig breeds. Indeed a genetic marker associated with the oestrogen receptor gene (ESR) of the Meishan has been shown to have beneficial effects on litter size and is described in WO92/18651.

The Booroola Merino breed of sheep is extremely prolific. Litter sizes of three or more are common. The significantly increased prolificacy of this breed has been shown to be due to the action of a single gene, FECB (for review see G W Montgomery, et al, *Endocrine Reviews*, 13: 309-328 (1992)). Genetic mapping using human DNA markers has shown that the human version of FECB is located on chromosome 4 (G W Montgomery, et al, *Nature Genetics*, 4: 410-414 (1993)) and is closely associated with the gene encoding secreted phosphoprotein-1 (SPP-1), also known as osteopontin (OPN), 2ar, bone sialoprotein-1, 44 kDa bone phosphoprotein and tumour secreted phosphoprotein. Comparative mapping (H Ellegren, et al, *Genomics*, 17: 599-603 (1993)) has shown that human

chromosome 4 and porcine chromosome 8 are highly similar (syntenic). The porcine SPP-1 gene is also located on chromosome 8.

5 More recently, it has been shown that a FECB-linked marker in cattle does not act as a marker for increased litter size in herds selected for increased ovulation rate (Blattman et al, *Mid-West Animal Science Meeting*, 18: 43 (1995)).

10

However, we have surprisingly found that, in pigs, certain DNA markers for OPN are associated with litter size, and thus can be used to select for pigs with a greater chance of producing increased litter size and to  
15 select against pigs which have alleles indicating smaller litter sizes. As used herein "increased litter size" means a significant increase in litter size above the mean of a given population.

20

It is interesting to note that there is an apparent break point in the chromosome synteny around OPN between sheep, cattle and man on the one hand and mouse and pig on the other (Montgomery et al, *J. Reproduction and Fertility supplement*, 49:113-121 (1995)). This suggests that the  
25 structure of the chromosome may be altered in this region, between animals having large litters (mouse and pig) and those with small litters (man, sheep and cow), such that the effect of the major gene for fecundity is modified. Possible explanations include the expression of  
30 the major gene may have been increased or decreased by being brought into a more transcriptionally active or inactive region; the major gene may have been brought directly under the control of an altered promoter element; the position of the major gene relative to OPN

may have been changed such that OPN becomes a more useable marker in assessing litter size potential in the pig than in sheep or cattle.

- 5 Thus, in a first aspect, the present invention provides a method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps:
- 10 (i) obtaining a sample of genomic DNA from a pig; and
- (ii) analysing the genomic DNA obtained in
- 15 (i) to determine which OPN allele(s) is/are present.

Suitably, step (ii), namely the determination of OPN alleles, is carried out by looking for particular DNA

20 markers linked either directly or indirectly to OPN.

Association between genetic markers and genes responsible for a particular trait can be disrupted by genetic recombination. Thus, the closer the physical distance

25 between the marker and the gene in question, the less likely it is that recombination will separate them.

It is also possible to establish linkage between specific alleles of alternative DNA markers and alleles of DNA markers known to be associated with a particular gene

30 (e.g. the OPN gene discussed herein), which have previously been shown to be associated with a particular trait. Thus, in the present situation, taking the OPN gene, it would be possible, at least in the short term, to select for pigs likely to produce larger litters, or

alternatively against pigs likely to produce smaller litters, indirectly, by selecting for certain alleles of an OPN associated marker through the selection of specific alleles of alternative chromosome 8 markers.

5 Examples of such markers known to be linked to OPN on porcine chromosome 8 include Sw61, Sw1085, Sw194, Sw16, SW790 and SO178, which markers are all microsatellites.

10 In a further embodiment of the invention a number of such markers are used. For example, pairs of markers might be utilised to bracket the major gene to reduce any possible effects of recombination. Examples of such combinations of markers include SO178 and SW61 and SO178 and SW790.

15 Since the effect may be related to the difference in gene orders of pigs (and mice) and sheep (and humans and cattle), this suggests that the most useful second marker will be in the non-homologous (non-syntenic) region of pig chromosome 8. An example of a suitable combination of

20 markers known to bracket this region would be OPN and SO178. However, the skilled man will appreciate that other useful markers could routinely be identified.

25 A particular genetic marker associated with OPN is a microsatellite. These are simple sequence repeats of 4, 3 or, more usually, 2 nucleotides, which occur essentially at random around the genome at approximately every 50,000 bases (about 60,000 microsatellites per haploid genome). Stuttering of DNA polymerase during

30 replication and unequal crossing-over during recombination are thought to result in the loss or gain of repeat units. This means that microsatellites are usually polymorphic and can have several repeat length alleles.

An example of a microsatellite associated with a given gene is  $(CA)_n$ , resulting in possible repeat unit length alleles, e.g.  $(CA)_2$ ,  $(CA)_9$ ,  $(CA)_{10}$ ,  $(CA)_{11}$  and  $(CA)_{12}$ .

5 Using primers capable of hybridising (for example, under stringent conditions) to regions flanking the microsatellite associated with the given gene, in combination with standard PCR techniques, PCR products of differing lengths can be generated, the length being  
10 dependent on the particular repeat unit length allele of the microsatellite.

Analysing the association of such PCR products using the microsatellite associated with the OPN gene with litter  
15 size has allowed marker length alleles associated with increased, and decreased, litter size to be determined in pigs.

Suitable primer pairs which will hybridise to flanking  
20 regions of such microsatellites include those having the following sequence:

GCTAGTTAATGACATTGTACATAA; or  
CCAATCCTATTTCACGAAAAAGC; and  
25 GTGTCATGAGGTTTTTCCACTGC; or  
CAACCCACTTGCTCCAC.

In particular, repeat unit length alleles for the above-noted microsatellite marker, designated 132 and 136, have  
30 been found to be associated with increased litter size in pigs. In addition, the repeat unit length allele, designated 112, has been found to be associated with reduced litter size in pigs.



In fact, the allele associated with increased litter size predominantly derived from a European parent stock. This is contrary to expectations since, as discussed above, the Meishan has four extra piglets per litter than either Landrace or Duroc, and it might have been expected that beneficial markers would have been associated with genes inherited from the Meishan parent stock.

In a second aspect the present invention provides a method of screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps:

- (i) obtaining a sample of genomic DNA from a pig;
- (ii) hybridising the genomic DNA from (i) with one or more suitable primers;
- (iii) carrying out one or more PCR cycles using the hybridised nucleic acid from (ii); and
- (iv) analysing the length of the PCR product obtained in (iii).

Suitably, the methods of the present invention are carried out using reagents and instructions presented in the form of a kit.

Thus, in a third aspect, the present invention provides a kit for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which comprises one or more

reagents or materials capable of identifying OPN alleles in a sample of pig genomic DNA.

5 A preferred kit of the invention will comprise reagents or materials capable of identifying alleles associated with DNA markers linked to the OPN gene, eg the microsatellite marker. Such a kit would most preferably comprise one or more DNA primers optionally together with standard PCR reagents.

10 Finally, the skilled person will realise that the methods and kits described herein can be used in conjunction with other already described methods and kits to screen pigs to determine those more likely to produce larger litters  
15 (or those less likely to). An example of such other methods and kits are those described in WO92/18651. It would, of course, be possible to produce combined kits which could be used to screen pig DNA using both methods.

20 In WO-A-9218651 and USSN 08/312312 there are disclosed methods for determining which pigs are more likely to produce larger litter sizes based on a linkage with the ESR gene. The skilled man will appreciate, therefore, that the screening methods of the present invention can  
25 be combined with the earlier disclosed ESR screening methods to provide a yet more powerful tool for such determinations. Thus, in a further aspect, the present invention provides a method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which  
30 method comprises the steps:

- (i) obtaining a sample of genomic DNA from a pig;

(ii) analysing the genomic DNA obtained in step (i) to determine which OPN allele(s) is/are present; and

5 (iii) analysing the genomic DNA obtained in step (i) to determine which allele(s) of at least one other gene linked to litter size in pigs is/are present.

10 In one preferred embodiment of this aspect of the invention the at least one other gene is the ESR gene, as described in WO-A-9218651 and USSN 08/312312.

15 In a final aspect the present invention provides a kit for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which comprises one or more reagents or materials capable of identifying OPN alleles in a sample of pig genomic DNA, together with one or more reagents or materials capable of identifying alleles of  
20 at least one other gene linked to litter size in pigs in a sample of pig genomic DNA.

Preferred features of each aspect of the invention are applicable to each other aspect *mutatis mutandis*.

25 The invention will now be described with reference to the following examples, which should in no way be construed as limiting the invention

EXAMPLE 1DNA Preparation

5 DNA can be prepared from any source of tissue containing  
cell nuclei, for example white blood cells, hair  
follicles, ear notches and muscle. The procedure  
outlined here relates to blood cell preparations; other  
10 tissues can be processed similarly by directly suspending  
material in K buffer and then proceeding from the same  
stage of the blood procedure. The method outlined here  
produces a cell lysate containing crude DNA which is  
suitable for PCR amplification. However, any method for  
15 preparing purified or crude DNA should be equally  
effective.

Blood should be collected in 50 mM EDTA pH 8.0 to prevent  
coagulation. 50  $\mu$ l of blood was dispensed into a small  
microcentrifuge tube (0.5 ml Eppendorf or equivalent).  
20 450  $\mu$ l of TE buffer was added to lyse the red blood cells  
(haem groups inhibit PCR) and the mix vortexed for 2  
seconds. The intact white and residual red blood cells  
were then centrifuged for 12 seconds at 13,000 g in a  
microcentrifuge. The supernatant was removed by gentle  
25 aspiration using a low pressure vacuum pump system. A  
further 450  $\mu$ l of TE buffer was then added to lyse the  
remaining red blood cells and the white blood cells  
collected by centrifugation as before. If any redness  
remained in the pellet, this process was repeated until  
30 the pellet was white. After removal of the last drop of  
supernatant from the pelleted white blood cells, 100  $\mu$ l  
of K buffer containing proteinase K was added and the  
mixture incubated at 55°C for 2 hours. The mixture was  
then heated to 95-100°C for 8 minutes and the DNA lysates

stored at -20°C until needed.

**Reagents**

5 TE buffer: 10 mM TRIS-HCl pH 8.0  
1 mM EDTA

10 K buffer: 50 mM KCl  
10 mM TRIS-HCl pH 8.3  
2.5 mM MgCl<sub>2</sub>  
0.5% Tween 20

Prior to use for lysates 10 µl of 20 mg/ml proteinase K (Boehringer Mannheim) per 1.0 ml of K buffer was added.

**15 PCR**

Reactions were set up as follows in thin walled 0.25 ml tubes (Perkin Elmer):

20 1.5 µl 10x buffer;  
1.5 µl 15 mM MgCl;  
1.5 µl 2 mM dNTPs (Pharmacia);  
0.5 µl of each primer at 5 mM (Genosys);  
9 µl sterile deionised water;  
0.1 µl (0.5 units) AmpliTaq DNA polymerase (Perkin  
25 Elmer);  
1 µl DNA lysate.

30 Reaction tubes were then placed on a Perkin Elmer 9600 thermal cycler and PCR carried out according to the regime indicated below:

94°C for 4 minutes;  
30 cycles of 94°C for 30 seconds, 58°C for 1 minute;  
and 72°C for 1 minute;

72°C for 4 minutes;  
4°C until required.

### Reagents

5	10x PCR buffer	100 mM Tris-HCl pH 8.3 (25°C), 500 mM KCl
	Forward primer	GCTAGTTAATGACATTGTACATAA
	or	CCAATCCTATTACGAAAAAGC
	Reverse primer	GTGTCATGAGGTTTGTGCCACTGC
10	or	CAACCCACTTGCTCCAC

If one of the primers is labelled with a fluorescent marker, the resulting products can be analysed on an automated DNA sequencer such as the Applied Biosystems 15 373 DNA Sequencer using Genescan and Genotyper software.

### EXAMPLE 2

## Polyacrylamide Gel Electrophoresis

20 5  $\mu$ l of the PCR products were mixed with 2  $\mu$ l of loading buffer and separated on a non-denaturing polyacrylamide slab gel in 1 x TBE buffer at 100V for 4 hours. The gel was then stained in a 50 ng/ml solution of ethidium bromide for 30 minutes and the PCR products visualised and photographed on a UV light transilluminator. PCR product sizes in base pairs were then estimated from relative mobilities as compared with known molecular weight markers run on the same gel. The size estimate of PCR products reflects the length of the microsatellite allele.

PCR products were also analysed on an Applied Biosystems DNA Sequencer following the use of a fluorescently

labelled primer in the PCR.

## **RESULTS**

### **5 OPN Allele Frequencies**

Results for OPN allele frequencies in different pig populations are presented in Table 1.

**TABLE 1 OPN Allele Frequencies in Different Pig Populations.**

Population	OPN Allele	Number	Percentage
Landrace	112	3	21
	132	6	43
	136	2	14
	142	3	21
Meishan	132	2	17
	140	8	67
	142	1	8
	154	1	8
L93	112	10	3
	122	2	1
	124	30	8
	132	39	10
	134	1	0
	136	36	9
	140	171	43
	142	60	15
	153	2	1
	154	43	11
L94	124	45	28
	132	15	9
	136	9	6
	140	84	52
	154	9	6

L93      Animals from a population founded by a Landrace x Meishan cross.

L94      Animals from a population founded by Duroc x Meishan cross.

***Statistical Analysis***

Female animals derived from L93 and L94 were scored for litter size (both total number born (TNB) and number born alive (NBA)), over several parities if possible, and these data were compared with OPN microsatellite genotypes for the same animal set. Statistical associations between litter size and OPN genotypes were investigated using the method of least squares to fit a general linear model. Least Squares Means (LSMs) for litter size were estimated for each OPN genotype. LSMs are the means adjusted for other effects in the model which could affect litter size.

The effect of individual OPN alleles was further dissected using an allele substitution model in which animals were classified into groups depending on whether they carried 0, 1 or 2 copies of a particular allele. LSMs for litter size were estimated for each group. The results for L93 are shown in Table 2.



TABLE 2 Allele Substitution Data for L93.

Number of Copies of Alleles									
0			1			2			
Allele	Trait	LSM	n	LSM	n	LSM	n	Model	OPN
112	TNB	13.0	306	11.8	21		0	+	+
	NBA	11.8		10.9				NS	NS
124	TNB	12.9	281	13.0	46		0	+	NS
	NBA	11.8		11.6				NS	NS
132	TNB	12.8	260	13.3	57	14.3	10	+	NS
	NBA	11.5		12.3		13.9		NS	+
136	TNB	12.9	271	12.9	53	17.5	3	*	+
	NBA	11.8		11.6		11.1		NS	NS
140	TNB	12.7	81	13.1	175	12.8	71	+	NS
	NBA	11.5		11.9		11.6		NS	NS
142	TNB	13.0	253	12.8	68	11.3	6	+	NS
	NBA	11.8		11.7		9.8		NS	NS
153	TNB	12.9	320	13.0	7		0	+	NS
	NBA	11.8		10.9				NS	NS
154	TNB	13.0	284	12.5	39	11.5	4	+	NS
	NBA	11.8		11.4		10.7		NS	NS

Significance level: \*,  $P < 0.05$ ; +,  $P < 0.10$ ; NS,  $P > 0.10$ .

Model includes season, AI or natural service, parity, generation and OPN genotype.

TNB total number born

NBA number born alive

LSM least squares means

n number of records, i.e. litters

It can be seen from the data that allele 112 appears to be related to a negative effect on litter size, whereas positive trends are seen for alleles 132 (NBA) and 136 (TNB). The data presented in Table 1 suggests that while alleles 112 and 136 were probably derived from the Landrace, allele 132 could have been derived from either the Landrace or Meishan ancestry. However, as the 132 allele is more than twice as common in the Landrace as the Meishan, it is likely that a significant proportion of the 132 alleles in L93 derive from the Landrace.

In order to investigate the potential of these alleles to act as predictors of litter size, additional data from L94 were included in the analysis. Allele 112 was not found in this line (presumably this allele is not found in Duroc). The combined data for alleles 132 and 136 are shown in Table 3.

TABLE 3

Number of Copies of Alleles									
		0		1		2		Significance of	
Allele	Trait	LSM	n	LSM	n	LSM	n	Model	OPN
132	TNB	12.2	375	12.9	82	14.2	12	***	+
	NBA	10.9		11.9		13.5		**	**
136	TNB	12.3	393	12.3	73	16.9	3	***	+
	NBA	11.1		11.0		10.5		NS	NS

Significance levels: \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; +,  $P < 0.05$ ; NS, not significant.

These data show that only allele 132 had a significant positive effect for litter size for both TNB and NBA. Although allele 136 was close to significance for TNB, it is probable that the effect here is due to a small amount of 136/136 animals (3) with very high observations.

The association between OPN allele 132 and high litter size has now been demonstrated in two different lines of pig (L93 and L94). This indicates that a QTL affecting litter size is closely associated with the porcine OPN gene. However, it is possible that in other families, lines or breeds of pig that a different OPN allele will be associated with increased litter size.

The results of a re-analysis of the data for L93 and L94 and for an additional line L07 (a large white line) is shown below in table 4 using an alternative model. This involved fitting each OPN allele as a variable and coding each animal with a 0, 1 or 2 for each allele (ie 0, 1 or 2 copies of each allele).

Fixed effects were herd-season-service type and parity. Sire was included as a random effect. ESR and OPN were fitted as covariables. All data per line were included, not just full- or half-sib families. OPN alleles with less than 10 litters of a second genotype were excluded from the analyses.

Traits analysed were total number born (TNB) and number born alive (NBA).

Three models were run for each line including the fixed, random and ESR effects as given above.

1. Model excluding OPN

2. Model including all OPN alleles
3. Model including OPN alleles individually

5        -2loglikelihood was obtained for each model. Significance  
of the model was calculated by subtracting the log  
likelihood from models 2 or 3 from model 1 and comparing  
the result against a Chi-squared distribution. Degrees of  
freedom (df) used was the difference between the two  
models.

10

The levels of significance per line for model 2 and any  
significant alleles in model 3 are given in the table  
below.

Table 4

Line	Model /allele	TNB		NBA	
		Signif- icance	Allele substit ution effect	Signif- icance	Allele substit ution effect
7	2	P<0.10		P<0.10	
	3/OPN122	P<0.05	-1.30	P<0.05	-1.37
93	2	P<0.10		P<0.05	
	3/OPN112	P<0.10	-0.87	P<0.10	-0.92
	3/OPN132	NS	+0.49	P<0.05	+0.72
	3/OPN154	P<0.10	-0.72	NS	-0.46
94	2	P<0.01		P<0.05	
	3/OPN124	P<0.10	-0.83	P<0.10	-0.74
	3/OPN132	P<0.01	+1.62	P<0.05	+1.42

The following conclusions can be drawn from this data:

1. OPN accounted for a significant amount of variation in litter size (after including ESR) for L07 (P<0.10); L93 (TNB: P<0.10; NBA: P<0.05) and L94 (TNB: P<0.01; NBA: P<0.05).

2. OPN allele 132 showed a significant positive effect on litter size in L93 and L94.

3. Other alleles OPN122 (L07), OPN112 and OPN154 (L93) and OPN124 (L94) showed significant negative effects.

EXAMPLE 3

5 Genomic DNA samples from a further line L03 (another large white based line) were obtained and analysed. The results are shown below in table 5. 416 animals with 1,010 litter records were analysed.

10 Several different models were run. All models included the effect of farm-month farrowed, parity and sire. ESR was fitted as a co-variate in all analyses.

Traits analysed were total number born (TNB) and number born alive (NBA).

15 Models used:

1. Total number born= farrow-month+sire+ESR+OPN allele
2. TNB or NBA= farrow-month+sire+ESR+OPN112+OPN122 etc

**Table 5**

Line	Model/Allele	TNB		NBA	
		Significance	Allele substitution effect	Significance	Allele substitution effect
5 03	1/OPN124	P<0.01	+0.72		
	1/OPN136	P<0.15	-0.27		
	1/OPN138	P<0.15	+2.04		
	1/OPN142	P<0.15	-0.27		
	2/OPN112	NS	+0.34	NS	+0.58
10	2/OPN122	NS	-0.13	NS	-0.18
	2/OPN124	<0.05	+0.65	NS	+0.33
	2/OPN132	NS	+0.31	NS	+0.08
	2/OPN136	NS	-0.29	NS	-0.37
	2/OPN138	<0.15	+2.06	NS	+1.81
15	2/OPN140	NS	-0.11	NS	-0.16
	2/OPN142	NS	-0.22	<0.15	-0.40
	2/OPN144	NS	-1.06	NS	-1.16
	2/OPN146	NS	+1.08	NS	+0.15
20	2/OPN154	NS	+0.02	NS	-0.02

25 This data indicates that OPN 124 shows a significant (P<0.01) positive effect for TNB of 0.7 for each copy of the allele. In addition, OPN 142 showed a trend toward a negative effect on litter size in L03, a similar effect to that seen for L93.

As discussed above, another gene ESR, has been shown to affect litter size in pigs and it is likely that other



genes linked with litter size will be identified in the future. We investigated whether certain beneficial allele combinations of the two separate genes, OPN and ESR, provide an additive effect on litter size.

5

To test this possibility we looked at the association between litter size and various combinations of ESR and OPN alleles. The results presented below in tables 4 and 5 show that indeed beneficial alleles of OPN can combine positively with beneficial alleles of ESR, such that an even greater litter size advantage can be realised than can be achieved through using beneficial alleles of OPN or ESR alone.

10

15 **Table 4:** Allele substitution effect for OPN and ESR markers on litter size (TNB) in line 93 (L93)

Marker	Allele substitution effect for TNB	Significance
OPN 132	+0.49	ns
ESR B	+0.34	ns
OPN 132 or ESR B	+0.39	P<0.1

20

**Table 5:** Expected litter size (TNB) advantage for various combinations of OPN and ESR markers in line 93 (L93) based on data presented in Table 4

ESR	ESR	Genotype		Litter size effect (TNB)
		OPN	OPN	
-	-	-	-	0.00
B	-	-	-	+0.34
B	B	-	-	+0.68
-	-	132	-	+0.49
-	-	132	132	+0.98
B	-	132	-	+0.83 (+0.78)
B	B	132	-	+1.17 (+1.12)
B	-	132	132	+1.32 (+1.27)
B	B	132	132	+1.66 (+1.56)

Litter size effects assume complete additivity (OPN 132= +0.49; ESR B= +0.34) except those in brackets which assume the effect of OPN 132 or ESR B= +0.39.

CLAIMS

1. A method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps:

(i) obtaining a sample of genomic DNA from a pig; and

(ii) analysing the genomic DNA obtained in (i) to determine which OPN allele(s) is/are present.

2. A method as claimed in claim 1 wherein the determination of OPN alleles in step (ii) comprises determining the presence of at least one allele associated with at least one DNA marker linked either directly or indirectly to OPN.

3. A method as claimed in claim 2 wherein the DNA marker is a microsatellite.

4. A method as claimed in claim 3 wherein the DNA marker is Sw1085, Sw194, Sw16, Sw790, Sol78 or Sw61.

5. A method as claimed in claim 4 wherein one or more primers capable of hybridising to a region associated with the microsatellite are added to the sample of genomic DNA followed by one or more cycles of PCR to generate primer extension products.

6. A method as claimed in claim 5 wherein the OPN allele or alleles present in the sample of genomic DNA is

determined by reference to the length of the primer extension product(s).

7. A method as claimed in claim 5 or claim 6 wherein  
5 one or more of the following primers are employed:

GCTAGTTAATGACATTGTACATAA;  
CCAATCCTATTCACGAAAAAGC;  
GTGTCATGAGGTTTTTCCACTGC; or  
10 CAACCCACTTGCTCCCAC.

8. A method of screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises  
15 the steps:

- (i) obtaining a sample of genomic DNA from a pig;
- 20 (ii) hybridising the genomic DNA from (i) with one or more suitable primers;
- (iii) carrying out one or more PCR cycles using the hybridised nucleic acid from (ii);  
25 and
- (iv) analysing the length of the PCR product obtained in (iii).

9. A method as claimed in claim 8 modified by any one or more of the features of claims 2 to 4.

10. A method as claimed in claim 9 wherein one or more of the following primers are employed:

GCTAGTTAATGACATTGTACATAA;  
CCAATCCTATTACGAAAAAGC;  
GTGTCATGAGGTTTTTTTCCACTGC; or  
CAACCCACTTGCTCCCAC.

5

11. A kit for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which comprises one or more reagents or materials capable of identifying OPN alleles in a sample of pig genomic DNA.

10

12. A kit as claimed in claim 11 which comprises reagents or materials capable of identifying alleles associated with DNA markers linked to the OPN gene.

15

13. A kit as claimed in claim 12 wherein the DNA marker is a microsatellite and the kit comprises one or more DNA primers capable of hybridising to a region of the genomic DNA associated with the microsatellite.

20

14. A kit as claimed in claim 13 wherein one or more of the following primers are included:

GCTAGTTAATGACATTGTACATAA;  
CCAATCCTATTACGAAAAAGC;  
GTGTCATGAGGTTTTTTTCCACTGC; or  
CAACCCACTTGCTCCCAC.

25

15. A kit as claimed in claim 13 or claim 14 which includes standard PCR reagents.

30

16. A method of determining which allele or alleles for a DNA marker associated with the pig OPN gene is/are associated with larger litter size, which comprises the

steps of:

- 5 (i) obtaining genomic DNA from one or more pigs;
- (ii) determining which allele or alleles are present for a particular DNA marker associated with the OPN gene;
- 10 (iii) comparing the result of step (ii) with a similar determination carried out for one or more pigs known to produce larger litter sizes.
- 15 17. A method for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which method comprises the steps:
- 20 (i) obtaining a sample of genomic DNA from a pig;
- (ii) analysing the genomic DNA obtained in step (i) to determine which OPN allele(s) is/are present; and
- 25 (iii) analysing the genomic DNA obtained in step (i) to determine which allele(s) of at least one other gene linked to litter size in pigs is/are present.
- 30 18. A method as claimed in claim 17 wherein the at least one other gene is the ESR gene.
19. A method as claimed in claim 17 or claim 18 modified by any one or more of the features of any one of

claims 2 to 7.

20. A kit for screening pigs to determine those more likely to produce larger litters, and/or those less likely to produce larger litters, which comprises one or more reagents or materials capable of identifying OPN alleles in a sample of pig genomic DNA, together with one or more reagents or materials capable of identifying alleles of at least one other gene linked to litter size in pigs in a sample of pig genomic DNA.
21. A kit as claimed in claim 20 wherein the at least one other gene is the ESR gene.
22. A kit as claimed in claim 20 or claim 21 modified by one or more of the features of any one of claims 12 to 15.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 96/01408

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/68 C07H21/04 C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 18651 (IOWA STATE UNIVERSITY RESEARCH) 29 October 1992 cited in the application see the whole document ---	18-22
A	SCIENCE , vol. 263, 25 March 1994, pages 1771-4, XP002018359 ANDERSSON, L., ET AL.: "Genetic mapping of quantitative trait loci for growth and fatness." -----	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

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- "E" earlier document but published on or after the international filing date
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Date of the actual completion of the international search

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9218651	29-10-92	AU-B- 660994	13-07-95
		AU-A- 1917692	17-11-92
		BR-A- 9205918	08-11-94
		CA-A- 2108579	20-10-92
		EP-A- 0580767	02-02-94
		JP-T- 6506837	04-08-94
		US-A- 5550024	27-08-96
		US-A- 5374526	20-12-94
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